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Evaluation of A-1 Medium for the Rapid Recovery of Fecal Coliforms from Marine Waters

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EVALUATION OF A-1 MEDIUM FOR THE RAPID RECOVERY
OF
FECAL COLIFORMS FROM MARINE WATERS

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ABSTRACT

The efficiency of the A-1 medium in the recovery of fecal coliforms from marine waters was compared with the APHA Standard Method MPN Test. The modified A-1 method with a 3 hour resuscitation period at 35°C was found to be as accurate and reproducible as the standard method in the determination of fecal coliform densities in marine waters. The direct A-1 method gave a much lower number of gas positive tubes but a higher percentage recovery of E. coli. The recovery of fecal coliforms from 168 marine water samples by the modified A-1 method was comparable to those obtained by the standard method. The specificity of the A-1 medium for E. coli was very good in natural marine waters. Klebsiella organisms in waters receiving pulp and paper wastes did, however, interfere with the specificity of the medium.

Resumé

L'efficacité du milieu A-1 pour recouvrir les colibacilles fécaux des eaux marines a été comparée avec la technique NPP normale de l'A.P.H.A. La technique A-1 modifiée avec une période de ressuscitation pour 3 heures à 35°C a été trouvée d'être aussi exact et reproductible que la technique normale pour déterminer les comptes de colibacilles fécaux dans les eaux marines. La technique A-1 direct a donnée un nombre beaucoup plus bas de cultures positives mais le pourcentage de ressuscitation d'E. coli était plus haut. La ressuscitation de colibacilles fécaux provenant de 168 échantillons d'eaux marines par la technique modifiée était comparable à la technique normale. La spécificité du milieu A-1 pour E. coli était très bonne dans les eaux marines. Cependant, la présence des organismes Klebsiella dans les eaux recevant les déchets d'usine de pâte et papier a gênée la spécificité du milieu.

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INTRODUCTION

There is a growing recognition and use of the fecal coliform group as an indication of fecal pollution in water (Geldrich, 1966). The fecal coliform test provides a more precise measure of the potential health hazard associated with recreational and shellfish growing waters than does the more ubiquitous total coliforms. For this reason, the USFDA has proposed and adopted a fecal coliform standard of 14 to be used as an adjunct to, or replacement of, the total coliform standard of 70 for the evaluation of shellfish growing waters (NSSP, 1974).

At present fecal coliform densities in seawater can be measured by the multi-tube MPN procedure and a membrane filtration technique. However, the inherent disadvantages associated with these procedures have prevented either one from achieving absolute acceptance by marine microbiologists. The multi-tube MPN procedure requires a maximum of 72 hours to complete and the precision of the result is relatively poor. The 95 percent confidence limits of the three dilution 15 tube test range from 31 to 289 percent of the absolute value plus an additional 23 percent positive bias factor⁽⁵⁾. In addition, the multi-tube MPN procedure is time consuming, requires considerable incubation space and large amounts of sterilized media and glassware. These requirements restrict the number of samples that can be processed. The membrane filter method, on the other hand, gives a more rapid, direct colony count with greater precision and requires less amount of incubation space, media and glassware, but recovers only a fraction of the fecal coliform population in a seawater sample. The low recovery exhibited by the membrane filter method is intrinsically associated with the immediate exposure of the fecal coliforms to an elevated selective temperature (44.5°C), and cell injury in a marine environment. Another problem associated with the membrane filter method is that some types of samples cannot be filtered because of high turbidity and suspended colloidal matter.

The fact that contaminated shellfish could serve as vectors for the transmission of enteric disease to humans make it desirable to develop a rapid method for the examination of shellfish and their growing waters for fecal contamination. The need is especially great in light of the ever increasing demand for their monitoring of shellfish growing areas in the Maritime Provinces. Various methods for the rapid determination of E. coli (a fecal coliform) in seawater and shellfish have been published. Andrew and Presnell (1972)⁽²⁾, demonstrated that a newly formulated A-1 medium gave E. coli recovery in 24 hours comparable to that of the standard methods procedure of the APHA. In the United Kingdom, the estimation of E. coli in shellfish is made by using a modified MacConkey agar incubated at 44°C. Qadri et al (1974), found that the recovery of E. coli in shellfish is enhanced by preincubation of MacConkey broth for two hours at 37°C and then 22-24 hours at 44°C. Apparently a considerable proportion of E. coli were able to acclimatize during the resuscitation period.

The purpose of this study was to evaluate rapid methods for the enumeration of fecal coliforms in shellfish growing area waters and to compare these with the conventional standard APHA method. The methods investigated were: (a) a 24 hour elevated temperature (44.5°C) test with A-1 medium, (b) A-1 modified method with preincubation of A-1 medium for three hours at 37°C, and (c) the 72 hour APHA standard method procedure.

2 MATERIALS AND METHODS

2.1 Sampling Collections

Approximately 10 seawater samples were collected every month for a period of one year from Bedford Basin, Nova Scotia (Figure 1). Water samples were collected in 200 ml sterile glass bottles at a depth of approximately one foot by means of a rod sampling device. All samples collected were kept in an insulated cooler and transported to the EPS microbiology laboratory at the Bedford Institute of Oceanography for analyses within two hours after collection. Water temperature and salinity were also determined for each sample.

2.2 Methods of Examination

2.2.1 Standard method. The five-tube decimal dilution (MPN) method, according to the APHA standard methods procedure for the bacteriological examination of seawater and shellfish,⁽¹⁾ was used. Bacto-lauryl Tryptose Broth was used as the presumptive test medium with incubation at $35 \pm 0.5^\circ\text{C}$ for 24 and 48 hours, and positive tubes were transferred to Bacto-EC medium and incubated in a water bath at $44.5 \pm 0.2^\circ\text{C}$ for 24 hours.

2.2.2 A-1 method. Three decimal dilutions of water sample were pipetted into each of five tubes of A-1 medium. The A-1 medium was prepared according to the formula of Andrews and Presnell⁽²⁾. The inoculated tubes were transferred immediately into a water bath maintaining a temperature of $44.5 \pm 0.2^\circ\text{C}$. Tubes showing any amount of gas after 24 hours incubation were recorded as positive.

2.2.3 Modified A-1 method. Water samples were pipetted directly into A-1 medium in three decimal dilutions using five tubes per dilution. The inoculated tubes were first incubated in an air incubator at 35°C for three hours before transferred directly into a water bath at $44.5 \pm 0.2^\circ\text{C}$ for 21 hours.

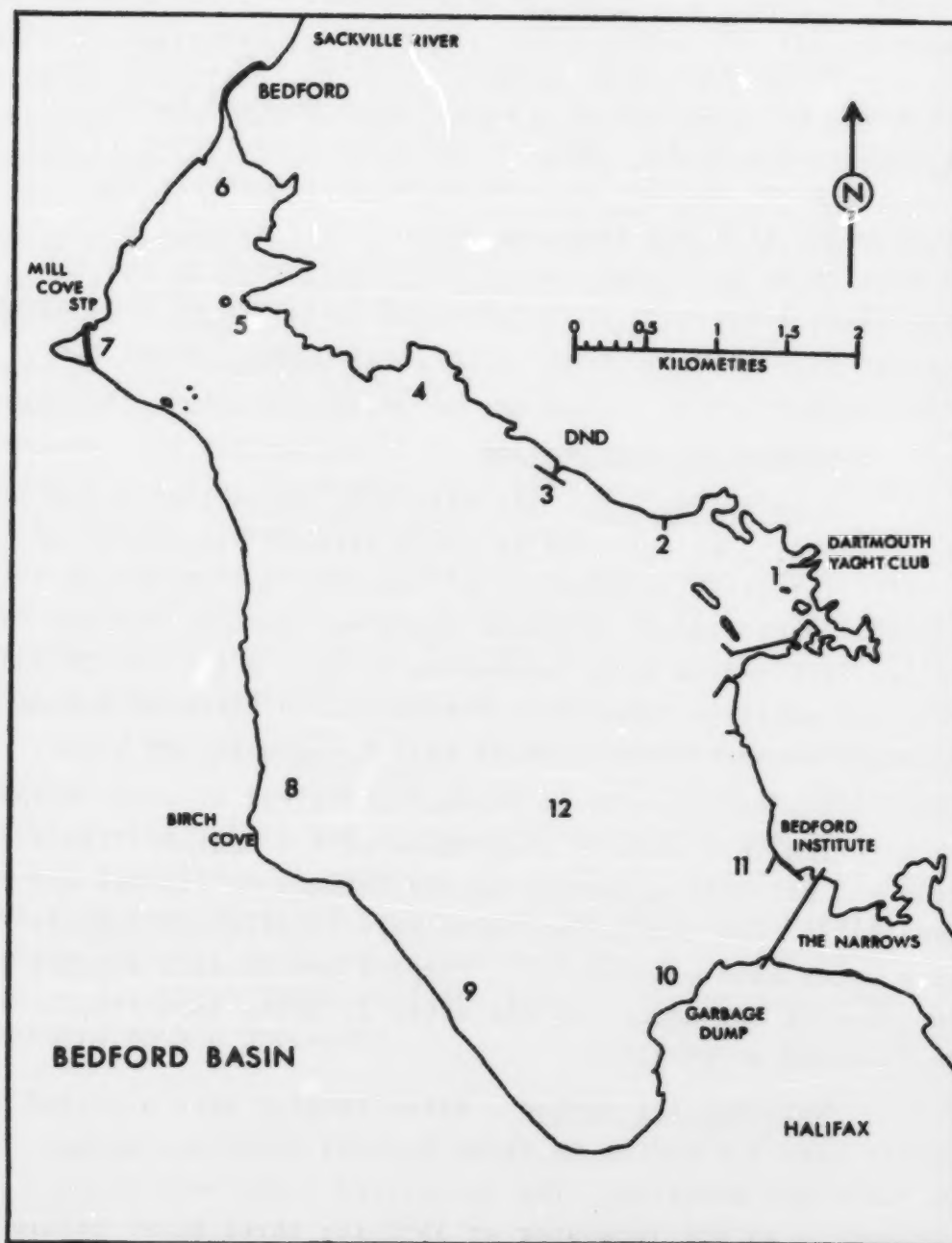


FIGURE 1 LOCATION OF SAMPLING STATIONS

2.2.4 Differentiation of the fecal coliform types. All positive tubes from representative test media were streaked on Levine eosin methylene blue (EMB) agar plates and incubated at $35 \pm 0.5^{\circ}\text{C}$ for 24 hours. Representative colonies of each type were picked and transferred to lactose broth and incubated at $35 \pm 0.5^{\circ}\text{C}$ for 24 to 48 hours. All cultures from gas-positive lactose tubes were subjected to the Indole, Methyl Red, Voges-Proskauer, Simons Citrate Agar, Motility Plate (20°C and 35°C) Ornithine Decarboxylase and hydrogen sulphide (TSI Agar) production tests.

3 RESULTS AND DISCUSSION

In evaluating a new medium the following characteristics of the method are considered.

3.1 Accuracy

The accuracy of A-1 medium was established by comparing the ability of the tested media to recover E. coli relative to that of the existing "standard method". A collaborative study was set up by the Food and Drug Administration to determine the comparative recovery of E. coli from a series of split artificial seawater samples using the standard fecal coliform test, the A-1 test and the modified A-1 test. Artificial seawater samples were prepared by adding salt to water to give a salinity of 15 parts per thousand and peptone was added to a level of 30 mg/l. The water was divided into three equal volumes and a pure culture of E. coli was added at three different levels. The levels of E. coli were added such that the expected recovery levels would be between 0-10 organisms per 100 ml at the low level, 10-100 organisms per 100 ml at the middle level and 100-1000 organisms per 100 ml at the high level. Two replicate subsamples at each of the three levels were sent to each of the 24 laboratories. Several laboratories received their samples too late to be analysed.

Table 1 shows geometric means for each method within each laboratory and across all laboratories. The data indicates there is no consistent difference between methods in the recovery of E. coli. An analysis of variance of the data is presented in Table 2. The calculation shows there is no significant difference between method means or laboratory mean recoveries. The results of the analysis of variance shows that the replicate subsampling error variance is 0.060. The expected variability of the 5-tube three dilution MPN test itself, when 10-fold dilution is used, is 0.060, indicating that the subsampling variability was totally accounted for by the variability of the MPN test.

TABLE 1 - RECOVERY OF FECAL COLIFORMS BY THE TESTED METHODS

LABORATORY	GEOMETRIC MEAN OF METHODS WITHIN EACH LAB. N=6 FOR EACH METHOD			GEOMETRIC MEAN LEVELS WITHIN EACH LAB. N=6 FOR EACH LEVEL			GEOMETRIC MEANS ALL LEVELS, ALL METHODS-N=17/LAB
	STANDARD	A-1	A-1 MODIFIED	LEVEL 1	LEVEL 2	LEVEL 3	
1	48	52	88	8	50	578	60
2	38	58	68	5	57	568	53
3	84	57	65	8	55	680	68
4	96	55	60	8	59	638	68
5	69	59	56	6	70	554	61
6	71	70	34	6	59	502	55
7	76	63	79	6	93	675	72
8	87	45	39	6	40	590	53
9	64	59	65	7	62	586	63
10	50	53	76	8	67	402	59
11	55	57	61	7	81	352	58
12	58	46	48	4	60	527	50
13	64	65	61	9	55	534	63
14	59	67	88	6	90	658	70
15	67	125	75	10	55	1110	85
16	59	35	41	4	56	382	44
17	74	106	62	8	83	740	79

TABLE 2 - ANALYSIS OF VARIANCE

EFFECT	SUM OF SQUARE	DEGREE OF FREEDOM	MEAN SQUARE	SIGNIFICANCE
Labs	1.278	19	0.067	N.S.
Methods	.1025	2	0.051	N.S.
Levels	211.21	2	105.6	**
Lab X Methods	2.416	36	0.067	N.S.
Levels X Methods	0.211	4	0.053	N.S.
Labs X Levels	1.892	36	0.053	N.S.
Labs X Levels X Methods	4.405	72	0.061	N.S.
Replicate Subsamples	10.251	171	0.060	N.S.

** Indicates significant difference between laboratory mean at $p < 0.01$

N.S. indicates no significant difference between methods at $p > 0.5$

3.2 Productivity

The adjusted X^2 method (Yates' correction) was used to determine whether the A-1 and modified A-1 tests were as productive as the standard procedure in fecal coliform determination in marine waters. X^2 is defined by the equation:

$$X^2 = \sum [(X_1 - \bar{X}) - 0.5]^2, \text{ where}$$

X_1 = number of samples giving higher counts in the standard method;

X_2 = number of samples giving higher counts in the A-1 test;

X_3 = number of samples giving higher counts in the modified A-1 test; and

$$\bar{X} = \frac{X_1 + X_2}{2} \text{ or } \frac{X_1 + X_3}{2} = \text{expected if both tests are equally productive.}$$

The adjusted X^2 value calculated from the examination of 168 marine water samples from Bedford Basin were 39.1 and 0.21 for A-1 and modified A-1 tests respectively. It may therefore be concluded that the standard method is much more productive than the A-1 test for fecal coliform recovery at a 0.001 level of significance. The modified A-1 test is as productive as the standard method for fecal coliform recovery in marine waters at 0.5 level of significance.

3.3 Comparability to Existing Method

The comparability of fecal coliform recovery by the A-1 test relative to that by APHA Standard Methods for the Examination of Seawater and Shellfish⁽¹⁾ was examined in 168 marine water samples collected from Bedford Basin. Table 3 shows that the standard method gave the highest recovery (49.1%) of fecal coliforms from marine water as defined by the number of positive tubes. The low recovery of fecal coliforms by the A-1 test appeared to be related to the "heat shock" effect on the immediate exposure of the fecal coliforms to the elevated temperature of 44.5°C. A three hour resuscitation period at lower temperature (35°C) enhanced the recovery of stressed

TABLE 3 - EVALUATION OF TEST MEDIUM FOR THE RAPID RECOVERY OF FECAL COLIFORMS IN SEAWATER

DATE	NO. OF SAMPLES	TEST	NO. OF TUBES INOCULATED	NO. OF POSITIVE TUBES	% OF POSITIVE TUBES	NO. OF ISOLATES	E. COLI TYPE	
							NO.	%
06/16/75	42	EC	630	273	43.3	40	34	85
		A-1	630	250	39.7	71	69	97.2
		MA-1	630	278	44.1	85	68	80
08/05/75	8	EC	120	94	78.3	19	17	89.5
		A-1	120	80	66.7	51	51	100
		MA-1	120	89	74.2	59	58	98.3
09/29/75	12	EC	180	104	57.8	45	28	62.2
		A-1	180	84	46.7	27	27	100
		MA-1	180	89	49.4	33	28	84.4
10/20/75	10	EC	150	93	62	37	33	89.2
		A-1	150	72	48	41	40	97.6
		MA-1	150	85	56.7	50	49	98
11/12/75	12	EC	180	76	42.2	31	27	87.1
		A-1	180	62	34.4	26	22	84.6
		MA-1	180	69	38.3	28	23	82.1
12/08/75	12	EC	180	139	77.2	52	45	86.5
		A-1	180	108	60.0	43	37	86.0
		MA-1	180	125	69.4	50	45	90.0
01/06/76	12	EC	180	126	70.0	52	34	65.4
		A-1	180	111	61.7	48	48	100
		MA-1	180	135	75.0	58	53	91.4
02/09/76	10	EC	150	62	41.3	44	32	72.7
		A-1	150	43	28.7	32	31	96.9
		MA-1	150	46	30.7	39	37	94.9
03/08/76	10	EC	150	74	49.3	51	22	43.1
		A-1	150	49	32.7	34	27	79.4
		MA-1	150	50	33.3	37	34	91.7
04/12/76	10	EC	150	42	28.0	25	13	52.0
		A-1	150	19	12.7	14	11	78.6
		MA-1	150	28	18.7	19	11	57.9
05/15/76	10	EC	150	41	27.3	27	17	63.0
		A-1	150	26	17.3	20	19	95.0
		MA-1	150	31	20.7	26	23	88.5
06/10/76	10	EC	150	41	27.3	19	19	100
		A-1	150	35	23.3	19	19	100
		MA-1	150	47	31.3	22	21	95.5
07/16/76	10	EC	150	88	58.6	44	31	70.5
		A-1	150	85	56.7	40	34	85.0
		MA-1	150	90	60.0	44	35	79.5
	168	EC	2,520	1,253	49.7	486	352	72.4
		A-1	2,520	1,024	40.6	466	435	93.3
		MA-1	2,520	1,162	46.1	550	485	88.2

EC = STANDARD METHOD EC TEST; A-1 = A-1 TEST; MA-1 = MODIFIED A-1 TEST

coliform cells from 40.6% to 46.1%. The results of the analyses have also shown considerable variance in MPN value between the three methods. This variance was no doubt partly due to the statistical nature of the MPN test itself. For this reason, it was necessary to take into account the 95% confidence limits of the MPN test. In comparing the 95% confidence limits of the standard MPN test with those of the A-1 and modified A-1 methods, it was found that the A-1 MPN fell within the confidence limits of the standard MPN 79.8% of the time, and the modified A-1 MPN fell within the confidence limits of the standard test 91.1% of the time.

3.4 Specificity

The specificity of the three tested procedures for E. coli was tested by verifying the coliform biotypes isolated from all the gas positive tubes from each media. Table 4 shows that both the A-1 and modified A-1 tests gave a higher percentage recovery of E. coli than the standard method. The A-1 test recovered 93.3% of E. coli from 460 fecal coliform isolates, whereas the modified A-1 test recovered 88.2% of E. coli from 550 isolates. The standard method recovered only 72.4% E. coli from 486 isolates. The high percentage of E. coli in tubes of A-1 medium suggested that the A-1 medium was more selective for E. coli and had a lower incidence of false positive reaction than the standard procedure. When adjusting the number of gas positive tubes with the isolate data, the modified A-1 test was found to give the highest number of E. coli positive tubes (1025), followed by the standard method (907) and the A-1 test (955) respectively.

To determine whether the specificity of the three methods holds in marine waters receiving industrial wastes, twenty water samples were collected from the Little East River estuary and analysed for fecal coliforms by the three tested procedures. This river received industrial wastes from Anil Canada Limited which is a hardboard plant. It is evident from Table 4 that none of the three tested methods were specific for E. coli recovery in these waters. The

standard method recovered only 11.9% E. coli, whereas the A-1 and modified A-1 tests recovered 32% and 21.1% E. coli respectively. Klebsiella constituted a majority of the coliform biotypes in the positive tubes.

TABLE 4 - SPECIFICITY OF TEST MEDIA FOR E. COLI IN AN ESTUARY RECEIVING INDUSTRIAL WASTES

TESTS	NO. OF SAMPLE	% OF POSITIVE TUBES	% E. COLI	% KLEBSIELLA	% ENTEROBACTER
Standard Method	20	59.3	11.9	82.5	5.6
A-1	20	47.3	32.0	63.0	5.0
Modified A-1	20	58.7	21.7	65.1	13.2

CONCLUSIONS

The standard method recovered higher numbers of fecal coliforms as defined by the number of gas positive tubes than either the A-1 or modified A-1 test. However, the low number of gas positive tubes in the A-1 test was largely compensated for by the greater degree of E. coli recovery. When comparing the ratios of the number of tubes containing E. coli to the number of gas positive tubes, the percentages obtained by the modified A-1 test was significantly higher than those by the standard method. The higher overall recovery of E. coli by the A-1 test as compared with the standard method would suggest that the A-1 medium is more selective for E. coli and there is a lower incidence of false positive reactions exhibited in this medium.

The specificity of the A-1 medium for E. coli in natural marine waters was very good and far superior than the standard method. However, in marine waters receiving pulp and paper wastes, the specificity of both the A-1 and standard method for E. coli was very poor. The percentage recovery of E. coli could be as low as 12 percent. The problem appeared most acute in marine waters containing an abundance of Klebsiella organisms. However, considering the fact that some Klebsiella strains have been responsible for infections of the urinogenital and upper respiratory tract, and the majority of Klebsiella strains could fit the definition of fecal coliform, the A-1 test could still be considered valid as a measurement of fecal pollution. After all, the objectives of routine bacteriological examination of shellfish growing waters are to detect fecal pollution and to protect the public from harvesting contaminated shellfish.

In view of the inherent limitation of the MPN test as a whole, it is doubtful whether minor differences in the results obtained by the modified A-1 test and the standard method should weigh more heavily in the selection of standard

procedure for monitoring shellfish growing waters than other relevant aspects of the test, such as reproducibility, speed, convenience and cost. Moreover, the disparity between MPN values in both tests for most of the water samples tested was not significantly larger than discrepancies between results obtained with permissible variations of the standard dilution method. The modified A-1 test has shown to be an accurate and reliable method for the recovery of fecal coliforms from seawater. Furthermore, its compatibility with the standard method concurrent with the advantages of speed and simplicity provide a strong argument for accepting the modified A-1 test as an alternate standard procedure for routine bacteriological examination of shellfish growing waters.

5

RECOMMENDATIONS

On the basis of the data presented in this report and others obtained from regular shellfish growing area water surveys, it is recommended that the Maritime Standing Committee on shellfish adopt the modified A-1 test as an alternate standard procedure for monitoring shellfish growing area waters in the Atlantic Provinces for an interim period of two years until a more specific test has been developed for E. coli. However, in the shellfish growing areas which are influenced by industrial wastes, biochemical differentiating tests should be performed on some of the gas positive samples to verify the fecal coliform biotypes.

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